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Serological Relationships Among Strains of Tobacco Ringspot Virus and Detection of Seed-Borne Plant Viruses.

Ronald Hillin Brlansky
Louisiana State University and Agricultural & Mechanical College

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SEROLOGICAL RELATIONSHIPS AMONG STRAINS OF TOBACCO RINGSPOT VIRUS AND DETECTION OF SEED-BORNE PLANT VIRUSES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirement for the degree of
Doctor of Philosophy

in

The Plant Pathology Department

by
Ronald Hillin Brlansky
B.S., Texas A&M University, 1970
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May, 1977

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Candidate: Ronald Hillin Brlansky

Major Field: Plant Pathology

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Approved:

K. S. Deswardt
Major Professor and Chairman

James B. Traynham
Dean of the Graduate School

EXAMINING COMMITTEE:

M L Harn

Lowell L. Black

H C. Holcomb

R. J. Cimbalnik

John P. Chilton

Date of Examination:

December 21, 1976

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ABSTRACT

Serologically specific electron microscopy (SSEM) was used to categorize serological strains of tobacco ringspot virus (TRSV). SSEM involves the attachment of virus particles to an adsorbed film of antibodies on electron microscope grids. Eleven isolates of TRSV were compared using SSEM. The reaction of each isolate was tested with its own antiserum and with antisera of each isolate, respectively. Crude virus extracts were diluted 1:100 and antisera were diluted 1:5,000. Reaction times for antiserum adsorption and virus attachment were constant for all isolates examined. The grids were stained with uranyl acetate and viewed with an electron microscope. Electron micrographs were taken at a predetermined magnification from which virus particle counts were made, then comparisons were made of these numerical values. The eleven TRSV isolates were placed into seven distinct groups. The seven groups were 1) North Carolina (N.C.) strain 38; 2) N.C. strain 39, grape strain (ATCC PV No. 157), Louisiana soybean isolate, HM soybean isolate, and the blackberry isolate; 3) N.C. strain 72; 4) N.C. strain 87; 5) watermelon isolate (ATCC PV No. 125); 6) Eucharis mottle strain (ATCC PV No. 48); and 7) cherry strain.

Leaf tissue from a collection of diseased soybean plants whose symptoms resembled those produced by TRSV were used to inoculate TRSV indicator plants. Most inoculations failed to produce symptoms on the test plants. Attempts to detect TRSV, tobacco streak virus (TSV),

tomato ringspot virus (TomRSV), and tobacco necrosis virus (TNV) were unsuccessful with SSEM of these isolates. Mechanical transmissions and SSEM were successful with known isolates of these same viruses. To date, attempts to produce an antiserum to partially purified preparations and to view virus particles in crude plant extracts with SSEM were also unsuccessful. Apparently a disease of unknown etiology was involved which was seed transmitted in soybeans.

SSEM was used to detect virus particles in extracts of soybean seeds infected with TRSV, barley seeds infected with barley stripe mosaic virus (BSMV), and lettuce seeds infected with lettuce mosaic virus (LMV). The seeds were halved and one half was assayed for virus. The remaining infected seed halves were diluted with healthy seed material in ratios of 1:10, 1:100, 1:1,000, and 1:10,000 and assayed. Virus particles were detected in ratios of 1:1,000 with both TRSV and BSMV which represents a ratio of one infected seed per 1,000 healthy seeds. LMV was detected in ratios of one infected seed per 100 healthy seeds.

INTRODUCTION

Electron microscopy of plant viruses in crude extracts has been difficult and has had limited use. The leaf dip procedure (5) has been used with some degree of success in the determination of the morphology of virus particles. By this method, plant extracts are applied to plastic films on electron microscope grids which are then stained, dried, and examined with an electron microscope. This procedure is usually restricted to rod-shaped viruses because isometric viruses are very difficult to distinguish from cellular components. Several techniques have been developed for assaying viruses which combine electron microscopy with serology (1, 3, 4, 17). These procedures involve a mixture of virus and antiserum which, following a suitable reaction period, is applied to an electron microscope grid. Using high resolution electron microscopy, a positive serological reaction is indicated when antibody molecules are seen outlining the virions. With these techniques, cellular components and salts from the virus-antiserum reactions make viewing the particles difficult and since virus particles are not concentrated on the grid surface, the detection limits are approximately the same as leaf dip procedures.

Derrick (8, 9) described a technique for the detection and identification of plant viruses in crude extracts. This technique, termed serologically specific electron microscopy (SSEM), also combines the use

of electron microscopy with serology. Electron microscope grids coated with plastic films were floated on drops of virus antiserum diluted with buffer. Unadsorbed serum proteins were removed by floating the grids on drops of buffer or washing in a stream of buffer. These grids were then placed on drops of crude virus extracts for 1-24 hours and subsequently washed with buffer to remove cellular debris and salts. The grids were then stained and viewed with an electron microscope. Virus particles were attached and concentrated on grids containing antiserum specific to the virus. SSEM also was used as a quantitative assay for viruses (10) and as a method of monitoring virus concentration during systemic infection and purification (12). Derrick and Brlansky (6, 13) successfully have demonstrated the application of SSEM for detecting all morphological types of plant viruses as well as the corn stunt mycoplasma from crude plant extracts. Antiserum dilutions from 1:1,000 to 1:10,000 were routinely used and attached approximately the same number of virus particles (13). Mixed infections of viruses of different particle morphologies were assayed successfully on the same SSEM grid using a mixture of antisera.

Unrelated viruses can be separated on the basis of certain host reactions or serological tests. Strains of a virus also can be separated using either or both of these methods. Conventional serological techniques, such as immunodiffusion and microprecipitin tests, often have been used for strain separation. These tests make use of a mass of visible precipitate formed when virus and antiserum are mixed in

certain concentrations. Comparisons are based on the amount of precipitation formed when two or more viruses are cross-reacted with each other's antisera. Results from these types of comparisons at best conclude that two viruses are either closely related, distantly related, or unrelated. These types of comparisons may become less meaningful as a larger number of isolates are tested.

SSEM is a technique which may be useful in the separation of virus strains. The antigen-antibody reaction consists of two stages or phases. When virus particles are reacted with a specific antiserum, the first phase of the antigen-antibody reaction occurs with the attachment of virus particles to antibody molecules. The second phase occurs when a visible precipitation appears due to a combination of these individual reactions into a lattice structure. Conventional serological methods utilize the differences in the precipitins formed when viruses are cross-reacted with each others antisera. SSEM involves only the first stage of the antigen-antibody reaction in which the virus-antibody complex is formed. Results based on this stage may be more precise than those based on the mass of precipitation formed with conventional serological methods. Numerical values are obtained from particle counts of each virus tested with each of the antisera to the viruses being compared. Dilutions, reaction times, and temperatures for all reactions should be kept constant for comparisons of the numerical values. Preliminary evidence was shown by Derrick (11) with sugarcane mosaic virus (SCMV) that SSEM could be used to separate virus strains.

Gooding (14) used host reaction studies and cross protection tests along with cross-absorption and gel diffusion tests to separate natural serological strains of tobacco ringspot virus (TRSV). In these studies at least six natural strains of TRSV were found from over 100 isolates tested. TRSV strains have been found to cause a number of severe diseases in a wide variety of plants (18). TRSV causes a very destructive disease commonly called bud blight of soybean (Glycine max (L.) Merrill). Bud blight symptoms consist of a mild mottle that may or may not be present and a proliferation of the developing buds that produce few or no mature seeds. Infected plants are often smaller and if seeds are produced they may be borne one per pod. Although TRSV in soybeans has been found throughout the heavy soybean producing area of the United States, its occurrence has been rather sporadic (2). Losses due to TRSV infection may be severe at times. In Louisiana TRSV was first isolated from a number of soybean plants exhibiting the typical bud blight symptoms described (N. L. Horn personal communication). The virus isolated produced typical red lesions when inoculated to Vigna sinensis L. 'Early Ramshorn' and also gave precipitin lines when reacted with TRSV antiserum (ATCC PV AS 45).

In 1975 surveys were made of Louisiana soybeans to obtain a number of virus isolates. Soybean plants exhibiting symptoms typical of TRSV bud blight were collected. Initial attempts to determine the causal agent were unsuccessful.

TRSV has been shown to be transmitted in seed, by nematodes, and by aerial vectors (18). Transmission of TRSV through soybean seed has been considered unimportant as a means of developing epiphytotics of bud blight primarily since the virus has numerous weed hosts (2). These observations suggest that vectors may spread the primary source of virus from weeds into the field. Soybean seedlings infected by seed transmission often have mild or no virus symptoms. These types of plants could easily serve as source plants for virus vectors. It is not known whether the primary source of virus spread by vectors is from infected weeds or seedlings. A sensitive method for distinguishing strains might possibly solve such a problem.

It has been shown with lettuce mosaic virus (LMV) that seed transmission even in low amounts can be a primary source of virus and directly affect the amount of disease present in a field due to the presence of efficient vectors needed to spread the virus (21). With LMV as few as one seed in 1,000 may be enough to give damaging levels of infection.

Detection of seed-borne plant viruses is a rather difficult problem. Methods now used to determine virus infected seed include screening large numbers (up to 30,000) of seedlings from a given seedlot, assaying with local lesion indicator plants such as Chenopodium quinoa L. (15), conventional serology, and separation of lighter weight infected seeds in airstreams (20).

SSEM could prove to be a useful tool in detecting and measuring plant viruses in individual seeds and in determining the distribution of virus in various seed parts. The sensitivity of SSEM observed previously (13) suggested the technique may be of value in detecting and quantitating seed-borne viruses.

The present study was initiated to determine (i) if SSEM can be used to serologically compare isolates of TRSV and group them into a number of strains, (ii) what strains of TRSV may be present in Louisiana soybeans, (iii) if SSEM can be used to determine the unknown cause of soybean bud blight, and (iv) the potential of SSEM in detecting seed-borne viruses.

MATERIALS AND METHODS

Viruses and antisera. Tobacco ringspot virus (TRSV) isolates in this study included: the grape strain (American Type Culture Collection Plant Virus 157 (ATCC PV)), the watermelon isolate (ATCC PV 125), and the Eucharis mottle strain (ATCC PV 48). North Carolina (NC) strains NC-38, NC-39, NC-72, and NC-87 were provided by G. V. Gooding, North Carolina State University. Blackberry and cherry isolates were obtained from R. Stace-Smith, Canada Department of Agriculture, Vancouver, B. C. A soybean isolate was obtained from R. S. Halliwell, Texas A&M University and was designated HM. A Louisiana soybean isolate was provided by N. L. Horn, Louisiana State University. Three other TRSV soybean isolates were also collected in Louisiana. All TRSV isolates were inoculated to cowpea (Vigna sinensis L. 'Early Ramshorn') and were subsequently propagated in cucumber (Cucumis sativus L. 'National Pickling') as a source for antisera production and SSEM assays. TRSV infected soybean seed 'Dare' were collected from infected greenhouse plants that had been inoculated with the Louisiana soybean isolate. Barley seeds 'Vantage' infected with barley stripe mosaic virus (BSMV) were obtained from T. W. Carroll, Montana State University. Lettuce mosaic virus (LMV) infected lettuce seeds were a gift from T. A. Zitter, University of Florida, Belle Glade, FL.

Antisera were provided by G. V. Gooding to the TRSV strains NC-39, NC-72, and NC-87, and by R. Stace-Smith to the blackberry and cherry isolates. Antisera to the grape strain and to the type strain were ATCC PV AS 26 and ATCC PV AS 45 respectively. Antiserum to BSMV was obtained from R. J. Shepherd, University of California, Davis, and antiserum to LMV was provided by T. A. Zitter, University of Florida, Belle Glade. Antisera to all other isolates were made as follows: partially purified virus was prepared from systemically infected cucumber using the method of Steere (19) through the first cycle of differential centrifugation. Six intraperitoneal injections were given to white mice at weekly intervals. Each mouse was injected with 0.5 ml of virus and Freund's incomplete adjuvant mixed 1:1. Trial bleedings were made by tail clippings and drops of blood were placed in a drop of Tris-NaCl buffer (pH 7.2, 0.15 M NaCl). SSEM grids were prepared by floating grids on these drops. Terminal bleedings were made by cutting the throats of anesthesized mice.

Procedure for serologically specific electron microscopy. The procedure used was that of Derrick and Brlansky (13). Copper, 200 mesh, 74 μm, handle electron microscope grids were used throughout this work. Grids were prepared by dipping in a 1% solution of polybutene in xylene, air dried, then coated with a film of Parlodion using a 0.5% solution dissolved in amyl acetate. The grids were carbon-coated in a vacuum evaporator. The grids were floated on drops of antiserum diluted 1:5,000 with Tris buffer (0.05 M, pH 7.2) for 30 minutes at room

temperature (Fig. 1). Unadsorbed serum proteins were removed by floating the grids on drops of Tris buffer. Experimental grids were immediately floated on crude virus extracts. Control grids were floated on either normal rabbit serum or on antiserum to a virus serologically unrelated to the virus being tested. Grids for storage experiments were washed in distilled water, air-dried, and stored at either room temperature or at 4 C. Virus-infected leaf tissue extracts were prepared by grinding in a mortar and pestle with an extraction buffer of 0.05 M Tris, pH 7.2, containing 0.15 M NaCl. All leaf tissue assayed was diluted 1:100 with extraction buffer.

SSEM grids were floated on drops of diluted virus extracts for a reaction period of one hour (Fig.1). The grids were washed to remove cellular debris by floating three times on drops of extraction buffer containing 0.4 M sucrose, twice in extraction buffer, and twice in distilled water. The virus particles were stained by floating on a solution of 5% uranyl acetate in 50% ethanol for 3 minutes, followed by washing vigorously in 50% ethanol. Grids were then blotted and allowed to air dry. Attached virions were viewed with an Hitachi HU 11-A electron microscope.

Detection of viruses in seed. SSEM was used to detect virus particles in extracts of soybean seeds infected with tobacco ringspot virus, barley seeds infected with barley stripe mosaic virus, and lettuce seeds infected with lettuce mosaic virus. Seeds from virus-infected plants were halved and one-half was assayed for virus.

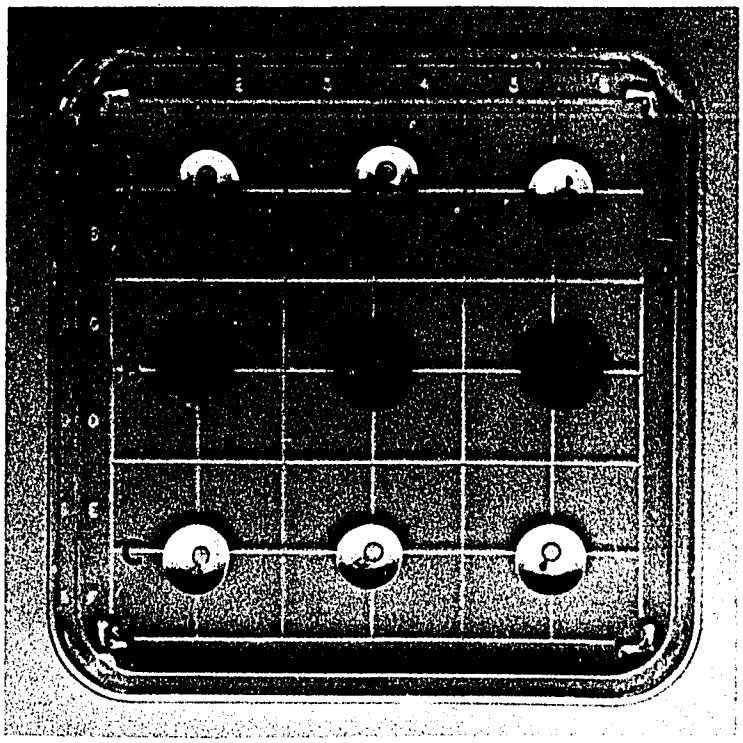

Fig. 1. Grids used in SSEM: A) Floating on drops of antiserum, B) Floating on drops of crude virus extracts, C) Floating on drops of wash buffer.

The seed halves were powdered in a mortar and pestle and then diluted with extraction buffer. Soybean and barley seed were diluted 1:10, but lettuce seed halves were diluted 1:1,000 to give enough material to test. The remaining seed halves from seed found to contain virus were mixed with healthy seeds in ratios of 1:10, 1:100, 1:1,000, and 1:10,000. These mixtures were ground with 9 volumes of extraction buffer and assayed.

Determination of TRSV strains using SSEM. SSEM grids were prepared using antiserum to each of the TRSV isolates being tested. Each virus isolate was reacted with its own antiserum (homologous reaction) and with antiserum specific to each of the other TRSV isolates (heterologous reaction). Micrographs of each virus-antiserum reaction were taken at a predetermine magnification. The total number of virus particles present on the 7x8 cm micrograph were counted. Numerical values of homologous and heterologous reactions were compared.

Studies to identify the unknown etiological agent of soybean bud blight. Tissue samples of soybean plants with typical TRSV bud blight symptoms were assayed using SSEM. All of the TRSV antisera produced in this laboratory and obtained elsewhere were tested. In addition assays were made using antisera to other viruses which included tobacco streak virus (TSV) red node strain (ATCC PV AS 50) and the HF strain (ATCC PV AS 6), tomato ringspot virus (TomRSV) (ATCC PV AS 25), and tobacco necrosis virus (TNV) (ATCC PV AS 72). The method of Steere (19) was used in an effort to obtain partially purified virus preparations and

attempts were made to produce an antiserum by injecting mice as previously described. SSEM was then performed with the bud blight material using the prepared antiserum.

Seeds from diseased plants were tested for seed transmission of bud blight in greenhouse plantings. Second generation seed also were tested for transmission.

RESULTS

Determination of serological relationships among strains of TRSV. All isolates of TRSV tested with SSEM were attached specifically with all of the TRSV antisera. Icosahedral virions, approximately 30 nm in diameter, were readily visible and distinguishable from cellular debris (Fig. 2). Both positive and negative stains were obtained with the use of uranyl acetate. The results of all homologous and heterologous reactions (Table 1) are presented as the total number of virus particles on a 7x8 cm electron micrograph taken at a magnification of 23,420X. Control SSEM grids prepared with bean pod mottle virus (BPMV) antiserum (ATCC PV AS 9) attached few or no virus particles (Fig. 3). In most cases, the homologous reactions represented the highest or one of the highest total number of virions attached. A typical comparison can be seen in Fig. 4 using the grape strain of TRSV and the Louisiana soybean isolate of TRSV. Comparisons between two strains were clearest using a 2x2 configuration representing both homologous and heterologous systems. The highest number of virus particles were attached with the homologous antiserum and the lowest number of particles with the heterologous antiserum. The differences between the homologous and heterologous reactions can be used to signify the amount of relatedness between two virus strains. These two viruses can be considered closely related due to small differences between the homologous and heterologous reactions.

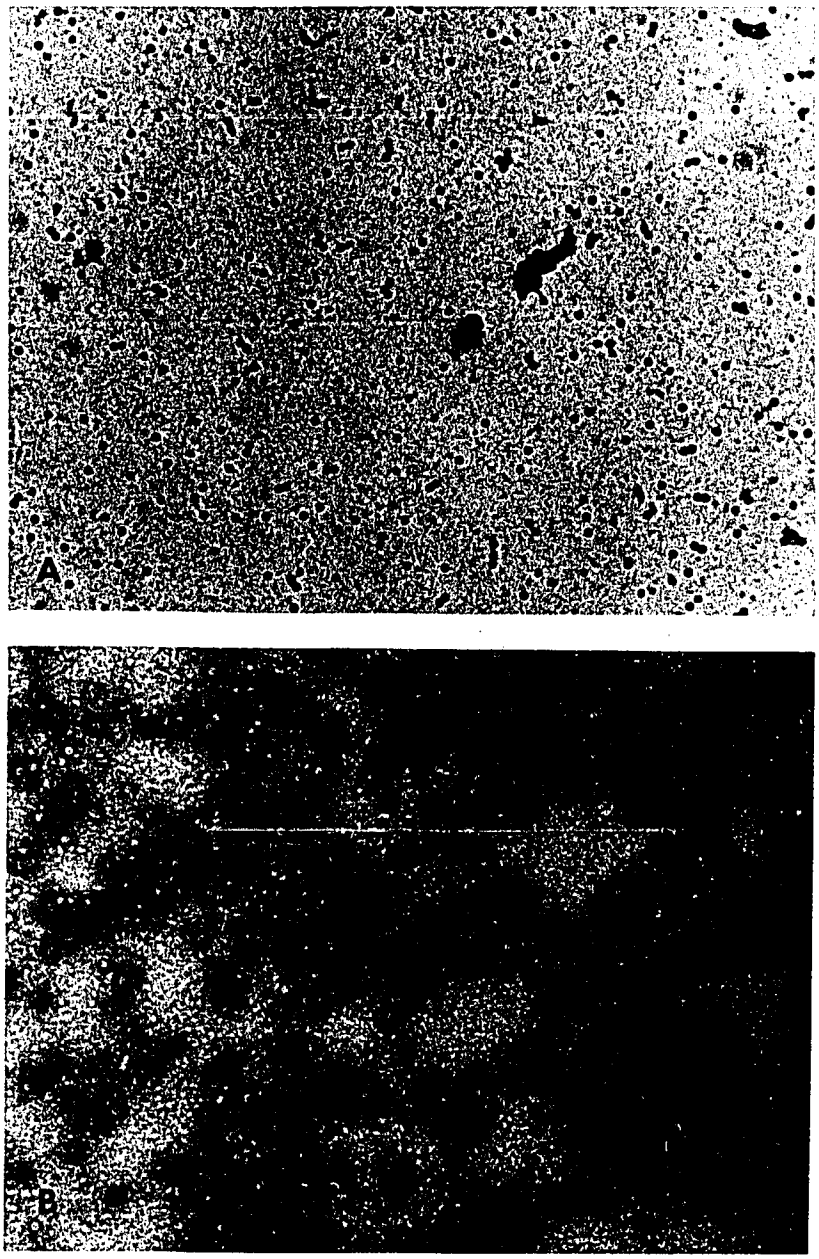

Fig. 2. Virus particles of the grape strain of TRSV specifically attached to the grapevine antiserum. A) Positive stain, B) Negative stain.

TABLE 1. Homologous and heterologous serologically specific reactions among strains of tobacco ringspot virus.

TRSV Strains	Antisera to TRSV Strains					
	Grapevine ATCC PV AS 26	Louisiana Soybean	NC-39	Blackberry	Cherry	NC-72
Grape Strain (ATCC No. PV 157)	912	752	904	832	1142	212
Louisiana Soybean Isolate (N. L. Horn)	1064	1136	1056	1224	1244	376
NC-39 Isolate (G. V. Gooding)	300	243	448	326	370	43
Blackberry Isolate (R. Stace-Smith)	941	443	501	1192	800	77
Cherry Isolate (R. Stace-Smith)	1116	225	1029	1496	2181	1236
NC-72 Isolate (G. V. Gooding)	626	311	568	896	1262	426

(cont.)

TABLE 1. Cont.

TRSV Strains	Antisera to TRSV Strains					
	Grapevine ATCC PV AS 26	Louisiana Soybean	NC-39	Blackberry	Cherry	NC-72
Watermelon Isolate (ATCC No. PV 125)	1112	417	808	952	1600	259
NC-87 Isolate (G. V. Gooding)	236	50	178	162	220	82
NC-38 Isolate (G. V. Gooding)	1512	533	1136	1080	1680	261
HM Soybean Isolate (R. S. Halliwell)	830	300	853	658	1023	307
Eucharis mottle strain (ATCC No. PV 48)	484	173	108	103	393	16

(cont.)

TABLE 1. Cont.

TRSV Strains	Antisera to TRSV Strains					
	Watermelon	NC-87	NC-38	HM Isolate	Eucharis Mottle	BPMV Ck. AS.
Grape Strain (ATCC No. PV 157)	120	49	78	181	135	0
Louisiana Soybean Isolate (N. L. Horn)	206	44	206	66	42	4
NC-39 Isolate (G. V. Gooding)	52	195	128	97	25	0
Blackberry Isolate (R. Stace-Smith)	117	240	132	792	18	0
Cherry Isolate (R. Stace-Smith)	152	70	176	305	142	5
NC-72 Isolate (G. V. Gooding)	89	62	35	264	14	0

(cont.)

TABLE 1. Cont.

TRSV Strains	Antisera to TRSV Strains					
	Watermelon	NC-87	NC-38	HM Isolate	*Eucharis* Mottle	BPMV Ck. AS.
Watermelon Isolate (ATCC No. PV 125)	133	46	16	230	22	0
NC-87 Isolate (G. V. Gooding)	98	278	323	10	15	0
NC-38 Isolate (G. V. Gooding)	25	45	497	110	27	3
HM Soybean Isolate (R. S. Halliwell)	141	48	158	943	35	3
Eucharis mottle strain (ATCC No. PV 48)	55	11	30	22	766	0

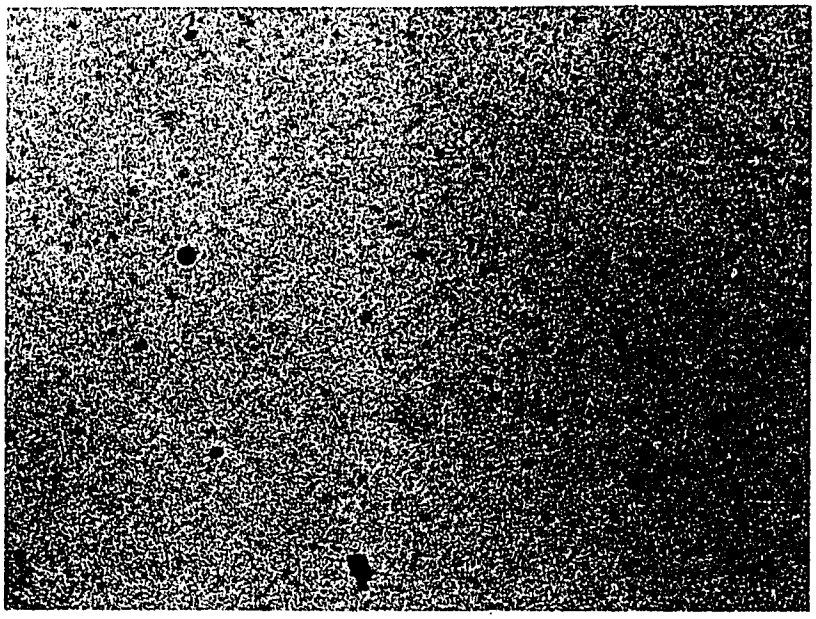

Fig. 3. Grape strain TRSV reacted on bean pod mottle virus antiserum.

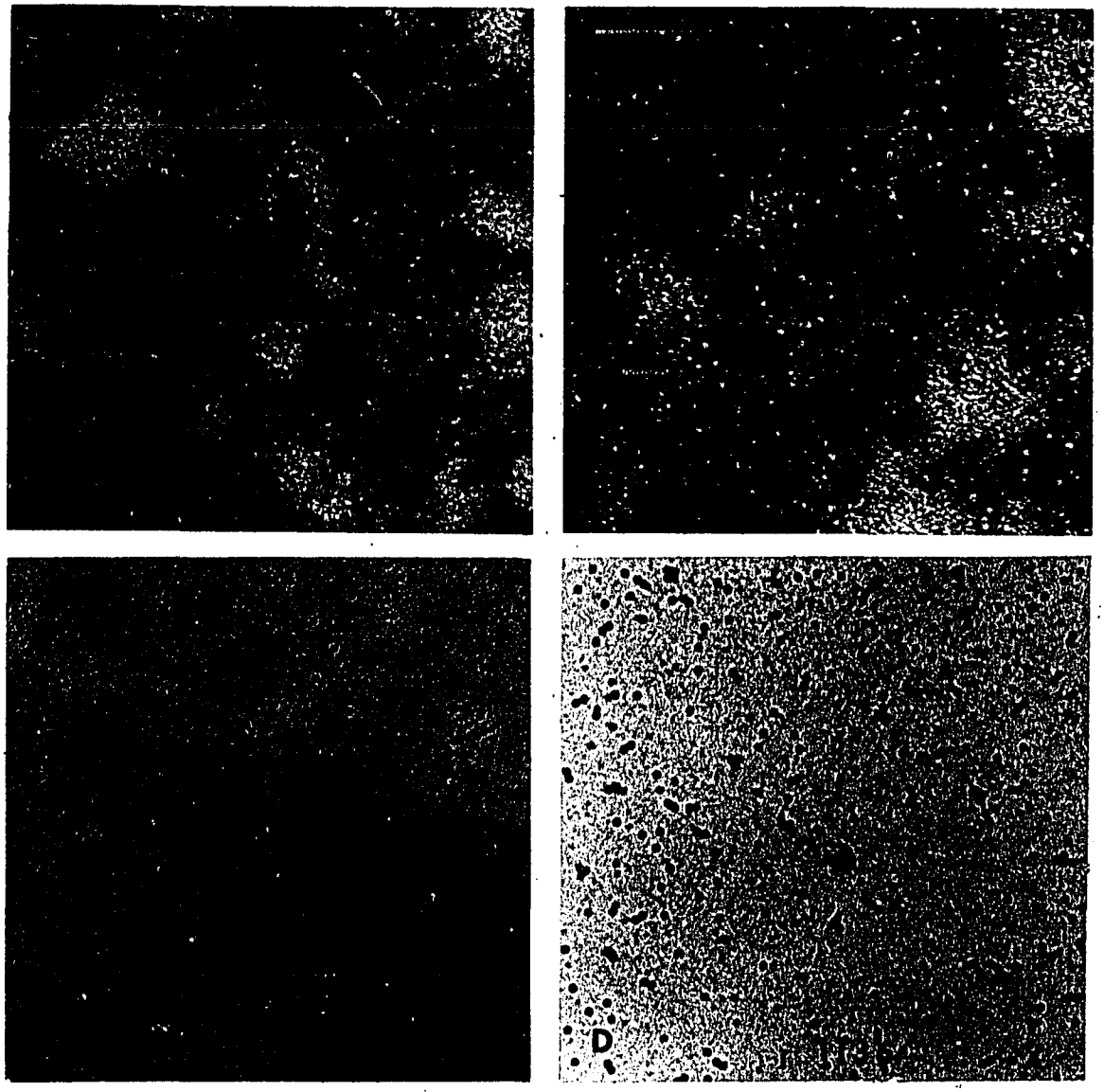

Fig. 4. Comparison of the homologous and heterologous reactions of the grape strain TRSV and the Louisiana soybean isolate TRSV. A-B) Grape strain on: A) grapevine antiserum, B) Louisiana soybean antiserum. C-D) Louisiana soybean isolate on: C) grapevine antiserum, D) Louisiana soybean antiserum. Numbers represent total particle counts from a 7x8 cm micrograph. 46,840X.

A comparison of the grape strain and the NC-39 isolate showed similar results (Fig. 5). Particle counts for the NC-39 isolate were lower but this was true for all the antisera reactions with this isolate. The homologous reactions of both isolates attached the greatest number of virus particles. Heterologous reactions when compared to homologous reactions again showed these isolates to be closely related serologically. Reactions involving the Louisiana soybean isolate and the NC-39 isolate when added to this scheme provided evidence that serological relatedness between them may be close when one heterologous reaction is compared with the homologous reaction. The other heterologous reaction however tends to question this since the particle number for NC-39 reaction with the Louisiana soybean antiserum dropped to 243 (Table 1).

Another type of reaction was that which showed serological separation. Comparisons of the reactions involving NC-72 and NC-87 isolates showed distinct serological separation between them. Heterologous reactions attached fewer particles than did homologous reactions (Fig. 6). Particle counts for the reactions between NC-39 and NC-87 isolates also showed the distinct serological separation of them with the use of SSEM (Table 1).

In some reactions serological relatedness or nonrelatedness were not as clear. Reactions such as the ones with NC-39 and NC-72 isolates and antisera were a good example of this (Fig. 7). Homologous reactions normally gave high particle numbers. The heterologous combinations produced two types of results: a high particle count with the NC-72

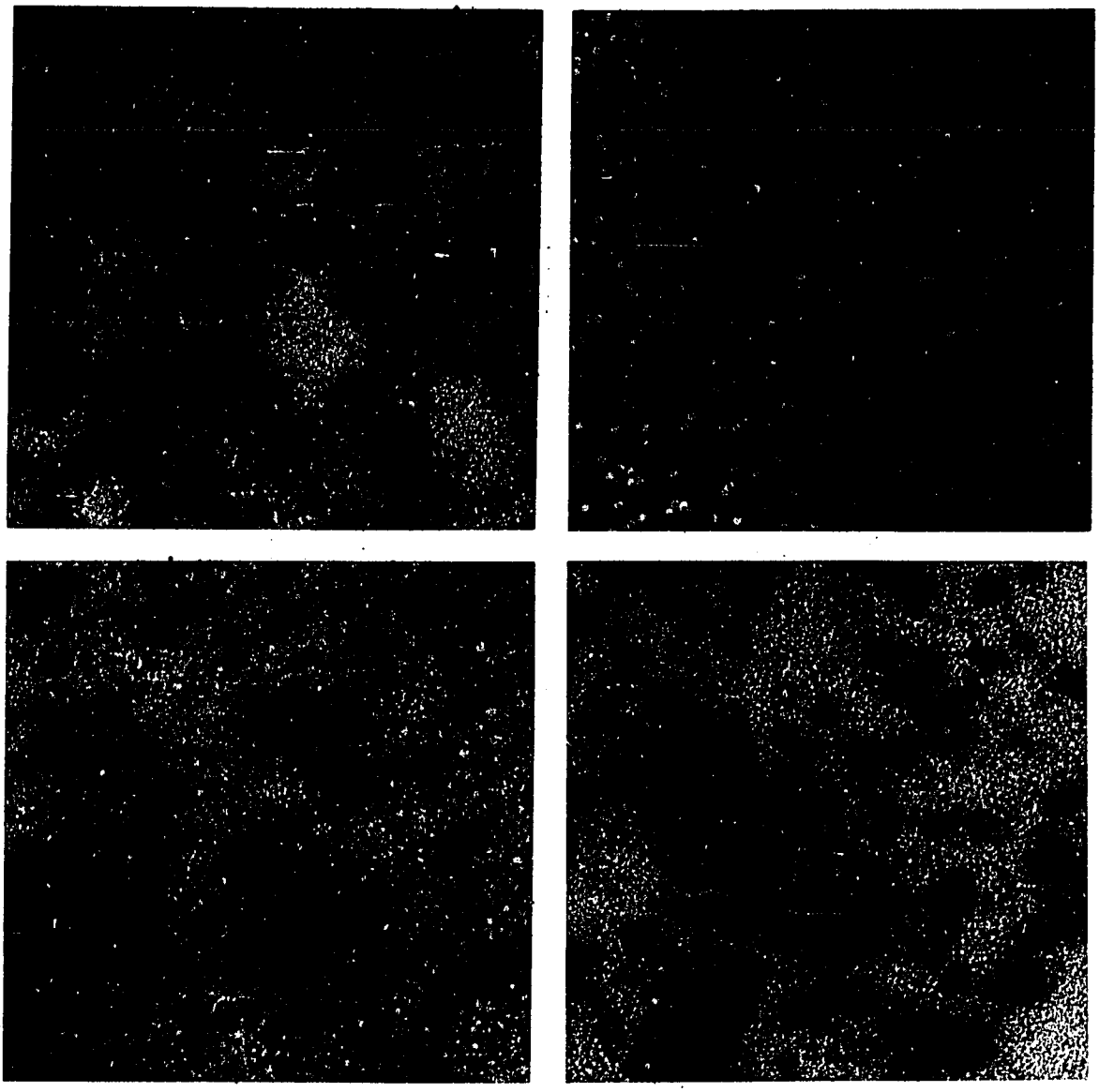

Fig. 5. Comparison of the homologous and heterologous reactions of the grape strain TRSV and the NC-39 isolate TRSV. A-B) Grape strain on: A) grapevine antiserum, B) NC-39 antiserum. C-D) NC-39 isolate on: C) grapevine antiserum, D) NC-39 antiserum. Numbers represent total particle counts from a 7x8 cm micrograph. 46,840X.

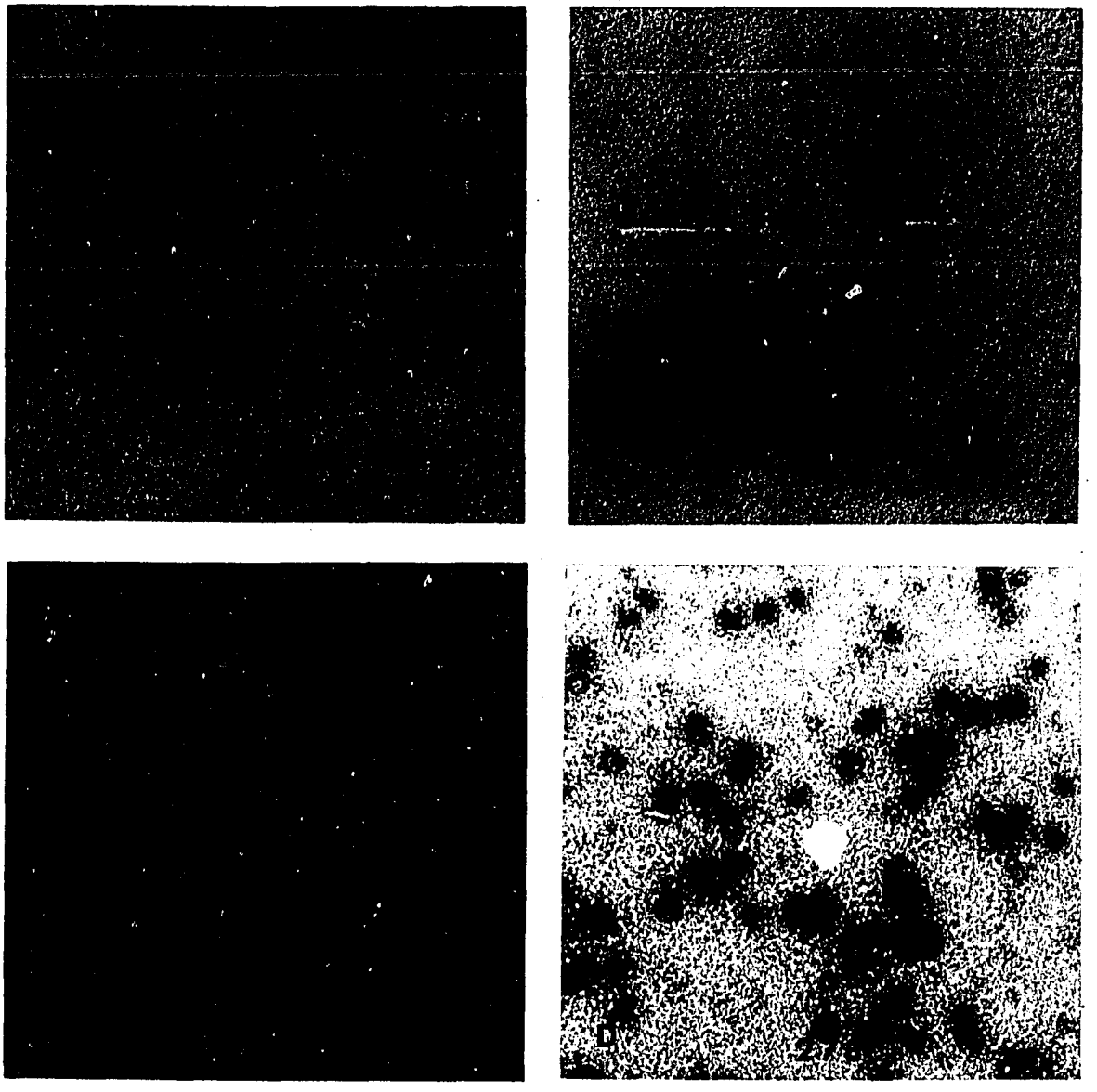

Fig. 6. Comparison of the homologous and heterologous reactions of the NC-72 and NC-87 isolates of TRSV. A-B) NC-72 isolate on: A) NC-72 antiserum, B) NC-87 antiserum. C-D) NC-87 isolate on: C) NC-72 antiserum, D) NC-87 antiserum. Numbers represent total particle counts on a 7x8 cm micrograph. 46,840X.

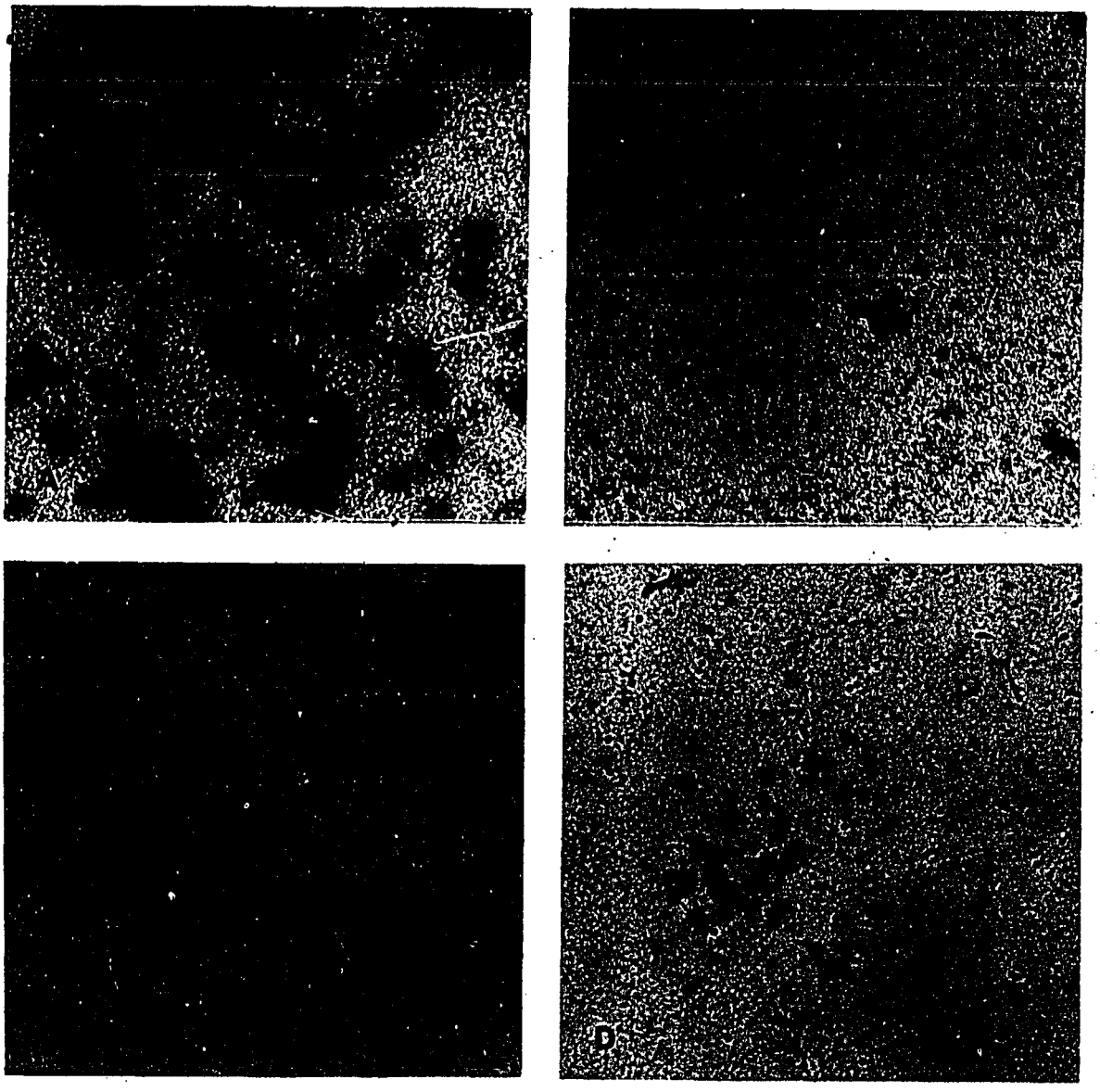

Fig. 7. Comparison of the homologous and heterologous reactions of the NC-39 and the NC-72 isolates of TRSV. A-B) NC-39 isolate on: A) NC-39 antiserum, B) NC-72 antiserum. C-D) NC-72 isolate on: C) NC-39 antiserum, D) NC-72 antiserum. Numbers represent total particle counts on a 7x8 cm micrograph. 46,840X.

isolate and the NC-39 antiserum and a low particle count with the NC-39 and the NC-72 antiserum. Similar results were found also in reactions involving the NC-72 and blackberry isolates. Both examples indicated serological separation since large differences were found between one heterologous reaction and its homologous reaction.

The watermelon isolate when compared with a number of isolates attached a lower number of virus particles when reacted with its homologous antiserum than when reacted with other antisera. The watermelon isolate antiserum was found to react weakly with most of the virus isolates tested. A second antiserum, again made by injecting mice with a partially purified virus preparation, also produced low numbers of attached virions. Antiserum and virus concentration differences were seen in many examples. The cherry isolate attached a large number of virus particles to most of the antisera and its antiserum likewise attached a large number of particles when reacted with most of the isolates. Serological separations were determined by comparisons of all homologous and heterologous reactions.

The three TRSV isolates obtained from Louisiana soybeans were found to react identically to the soybean isolate provided by N. L. Horn, Louisiana State University, when inoculated to soybean ('Dare'), cucumber ('National Pickling'), and cowpea ('Early Ramshorn'). SSEM results were the same as those reported with the Louisiana soybean isolate (Table 1).

Studies on the unknown cause of soybean bud blight. No virus particles were attached to any of the TRSV antisera specific grids when

reacted with crude extracts of the soybean bud blighted plants. Results using TSV antiserum, TomRSV antiserum, and TNV antisera also were negative. Attempts to specifically attach virus particles using an antiserum to a partially purified preparation of this tissue also were unsuccessful. This antiserum also failed to attach virus particles from cucumber plants known to be infected with the Louisiana soybean isolate of TRSV.

When seeds obtained from bud blighted plants were planted in the greenhouse the offspring developed the same type of symptoms. About 70-80% of the seeds obtained from bud blighted plants produced plants that were blighted and the condition was carried to the second generation. Attempts to mechanically transmit the unknown causal agent were unsuccessful.

Detection of seed-borne plant viruses. All three seed-borne viruses tested were successfully detected in half-seeds with the use of SSEM. Micrographs of each of the three different particle types detected are exemplified in Fig. 8. High concentrations of TRSV were found in soybean seed and BSMV in barley seed. The concentration of LMV was so low that only an occasional virus particle was obtained. When individual seeds were tested for virus, 75-80% of the soybean seeds contained TRSV, 40-42% of the barley seeds were infected with BSMV and 10% of the lettuce seeds contained LMV.

Virus particles were detected in both TRSV-infected soybean seed halves and BSMV-infected barley seed halves diluted 1:1,000 with healthy

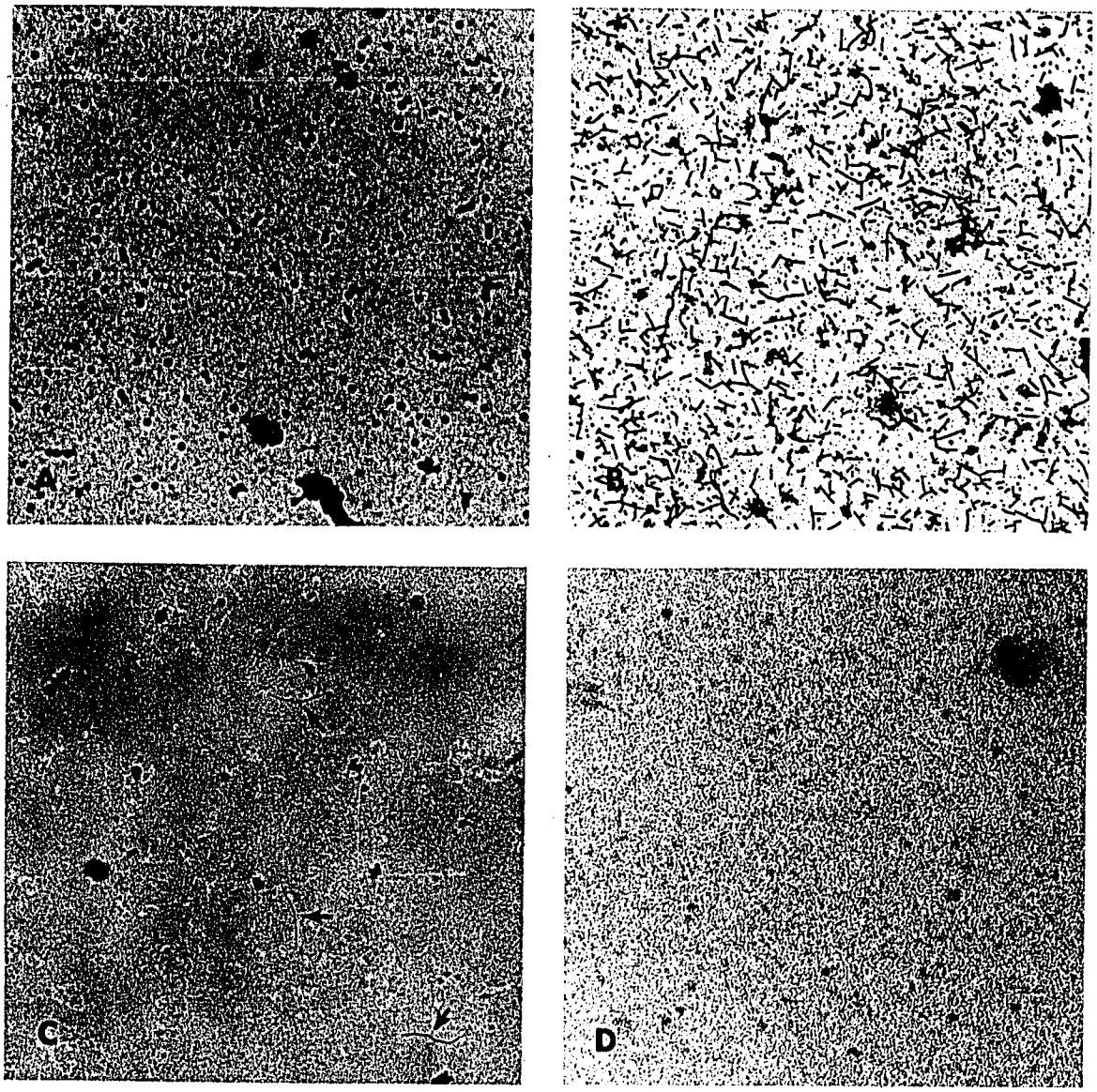

Fig. 8. Three types of viruses detected with SSEM from infected seeds. A) TRSV from infected soybean seed, 46,840X. B) BSMV from infected barley seed, 9,000X. C) LMV from infected lettuce seed, 9,000X. D) Check grid.

seed (Figs. 9, 10). These dilutions represented one infected seed per 1,000 healthy seeds. In terms of actual virus dilution both were detected at 1:10,000 since all mixtures were diluted 1:10 with extraction buffer. LMV was detected at 1:100 in mixtures tested (Fig. 11). This represented one infected seed per 100 healthy seed. The virus actually was diluted 1:1,000 because of dilution with buffer.

Storage of SSEM grids. SSEM grids prepared with antiserum to the Louisiana soybean isolate and the grape strain of TRSV, stored for up to 28 days at 4 C, were able to attach virus particles. Specific grids to both these isolates also were useable when air dried and stored at room temperature for 35 days (Fig. 12). Storage of BSMV antiserum coated grids for 35 days at room temperature (Fig. 12) did not effect its activity for use in serological tests.

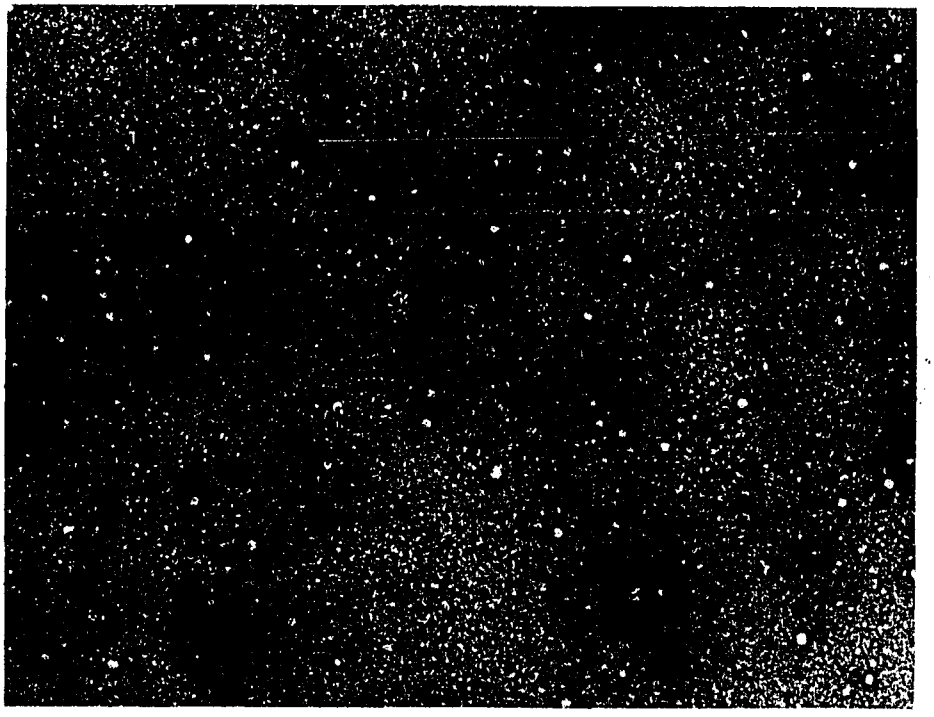

Fig. 9. TRSV detected in soybean seed at a dilution of 1:1,000 with healthy seed, 46,840X.

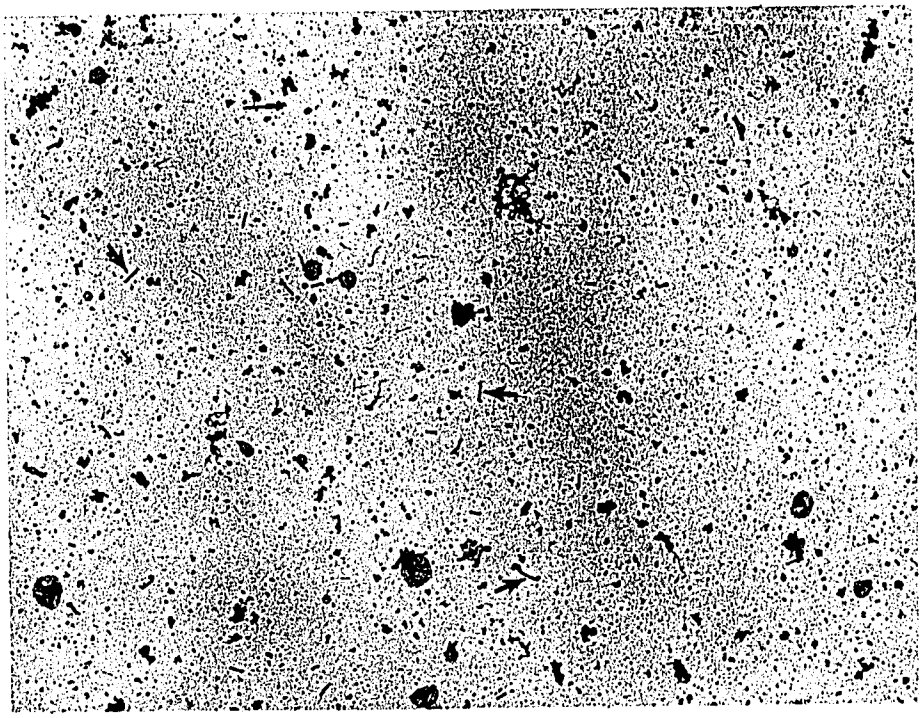

Fig. 10. BSMV detected in barley seed at a dilution of 1:1,000 with healthy seed. 9,000X.

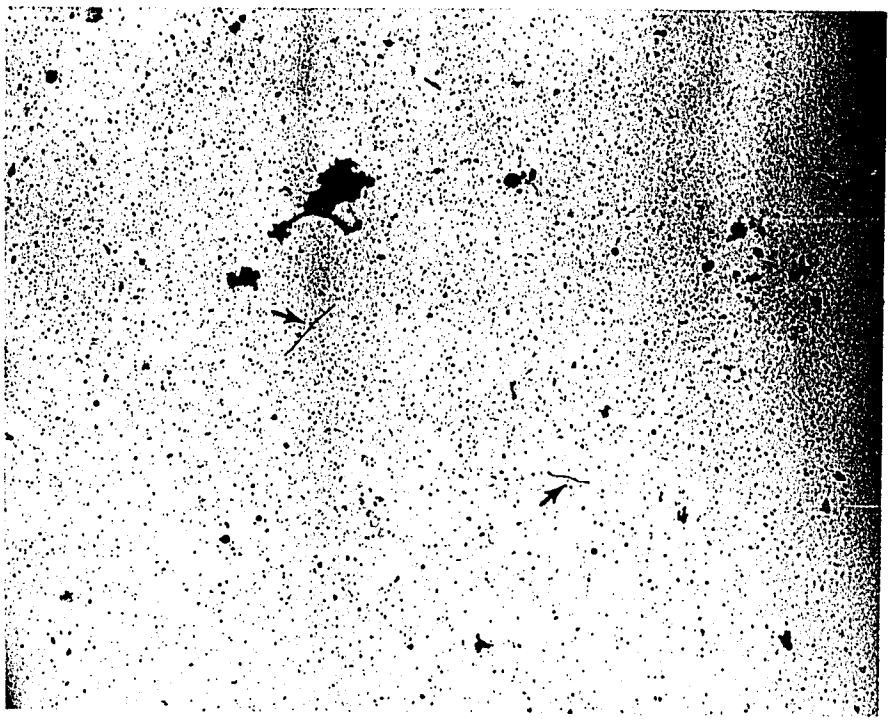

Fig. 11. LMV detected in lettuce seeds at a dilution of 1:100 with healthy seeds, 9,000X.

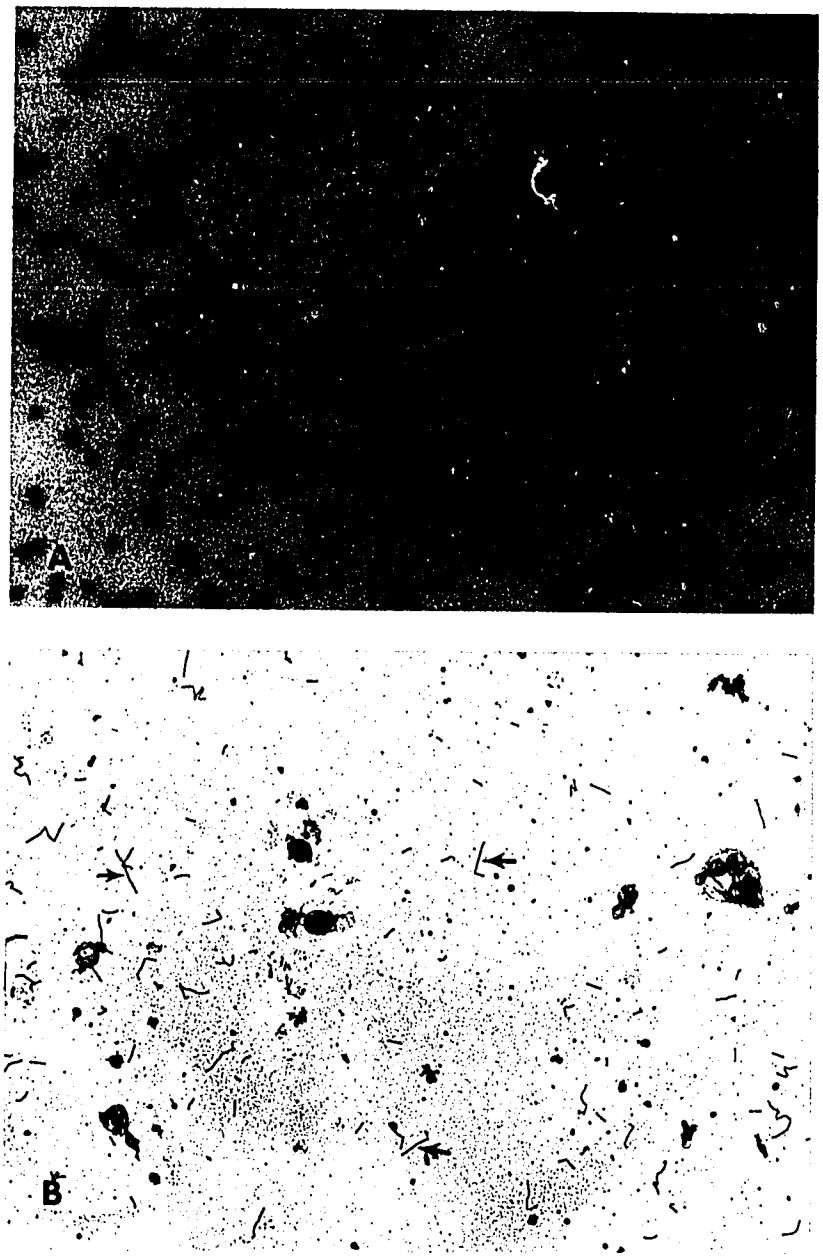

Fig. 12. Reactions with SSEM grids stored for 35 days at 24 C. A) TRSV, 46,840X. B) BSMV, 9,000X.

DISCUSSION

Serologically specific electron microscopy was found to be very useful in the separation of serological strains of TRSV. This technique provided a means for utilizing the initial phase of the antigen-antibody reaction which is the attachment of antigen with antibody. Most serological methods utilize the second phase of the antigen-antibody reaction, which occurs when a visible precipitation is formed. Tests utilizing this stage may produce misleading results under certain conditions due to host components that react with contaminating antibodies. Virus-antibody complexes which occur in small amounts may not produce strong enough precipitin lines or agglutinations to be detected by conventional serological methods.

Results from SSEM studies of TRSV isolates indicated that many different types of reactions may be produced. Two viruses are considered serologically related when the differences in the particle numbers between homologous and heterologous reactions involving these two viruses are very small. The reactions involving the grape strain and the NC-39 strain provide a good example of this. Particle numbers among homologous and heterologous reactions were not different. Two viruses were considered serologically different when heterologous particle numbers were considerably less than homologous reactions. An example of this was seen in the reactions involving NC-72 and NC-87 strains. A third

type of serological reaction occurred when one heterologous reaction produced particle numbers lower than its homologous counterpart but the other heterologous system attached more particles than its homologous reaction. The relationship between NC-39 and NC-72 strains is a typical example. These results may be due to antigenic determinants of one strain being recognized by the antibodies to the other, but those of the other strain may be not recognized by antiserum to the first strain. Gooding (14), who tested the reciprocal-absorption of TRSV strains, found that when a certain strain was used as an absorbing antigen with a particular antiserum, the antigen would absorb out antibodies specific for a number of other strains. For example, strain NC-72 was able to absorb out antibodies for itself and for all the other strains except those for the homologous strain. This indicated the special antigenic relationships that the NC-72 strain had with all the other TRSV strains.

The different types of serological reactions observed with SSEM also may be similar to certain types of reactions observed with agar-gel double diffusion. The precipitin line formed between two viruses and their respective antisera signifying an identity reaction may be expressed in SSEM as the similar homologous-heterologous particle counts. Attempts to correlate these types of reactions with SSEM results have not been made at this time.

In this study, seven groups of TRSV were separated serologically with the use of SSEM. Results indicate that all four NC-strains tested were serologically distinct. The watermelon isolate and the Eucharis

mottle strain also were found to be distinct strains of TRSV. These separations were identical to the results found by Gooding (14) using agar-gel double diffusion, cross absorption, and host reactions. The Louisiana soybean isolate and the grape strain were found to be closely related serologically to the NC-39 strain. The HM soybean isolate was related to the NC-39 strain also, but was distinct from the Louisiana soybean isolate and the grape strain. The blackberry isolate was closely related to both the grape strain and the Louisiana soybean isolate but distinct from the HM soybean isolate and the NC-39 strain. The cherry isolate was placed into a separate, distinct group.

SSEM was used successfully for the detection and identification of virus particles in extracts of soybean seeds infected with TRSV, barley seeds infected with BSMV, and lettuce seeds infected with LMV. These results showed the variety of virus types and the kinds of tissues that may be assayed with SSEM. The different amounts of starches and oils present in the seeds tested presented a number of problems in grinding the extracts and in removing the debris from reacted grids. Most of the debris on the grids was easily removed with successive washings in extraction buffer which contained 0.4 M sucrose. Successful assays were performed on half seeds. This amount of tissue was considerably smaller than most virus-infected plant tissue assayed with SSEM.

The results of the detection limit studies proved SSEM to be a very sensitive technique for the detection and identification of seed-borne plant viruses. The percentages of infected seeds found with each of the

viruses tested were consistent with known values (7, 16, 19). The success of this technique for detection of virus in apparently healthy seed material at dilutions of 1:1,000 (representing one infected seed per 1,000 healthy seeds) suggests its possible use in commercial and research areas for screening of seed lots for virus-infected seed. Virus detection in seeds would be of particular interest with viruses such as LMV that has low seed transmission yet requires only a very small percentage of infected plants in the field to cause extensive disease.

Storage of grids at room temperatures was preferred to storage at 4 C where copper grids corroded frequently. The fact that SSEM grids can be successfully stored at room temperature for 4 weeks or longer provides a convenient method for temporary storage of specific antiserum to certain viruses. Grids prepared in advance can be used in processing a large number of diagnostic samples.

Antisera to a number of different viruses that cause symptoms similar to those of Louisiana soybean bud blight failed to attach virus particles using SSEM. An attempt to prepare an antiserum and attach virus particles from crude extracts also was unsuccessful. However, these results did not prove that a virus was not the causal agent of these "false bud blighted" plants. However, the bud blight symptom was transmitted to the offspring through seed, which suggests the agent may be a virus.

The numbers of "false bud blighted" plants have become increasingly more numerous in soybean fields in recent years. SSEM and greenhouse

inoculation studies have shown that TRSV was not associated with many of the plants that exhibited the bud proliferation symptom. Since "false bud blight" appears to be a disease of potential importance and since the cause of it has not been resolved, it appears evident that additional research should be directed toward transmission studies. Determination of the mode of transmission would then specify what means might be used to determine the causal agent. Purification methods might then be formulated and attempts to produce an antiserum to the causal agent may be done. After production of an antiserum, SSEM could be used to detect and identify the causal agent of "false bud blight".

LITERATURE CITED

1. ALMEIDA, J. D., and A. P. WATERSON. 1969. In K. M. Smith and M. A. Lauffer, eds. Advances in Virus Research Vol. 15:307-308. Academic Press, New York, 1969.

2. ATHOW, K. L., and J. B. BANCROFT. 1959. Development and transmission of tobacco ringspot virus in soybean. Phytopathlogy 49: 697-701.

3. BALL, E. M. 1971. Leaf-dip serology. In K. Maramorosch and H. Koprowski, eds. Methods in Virology Vol. 5:445-450. Academic Press, New York, 1971.

4. BALL, E. M., and M. K. BRAKKE. 1968. Leaf-dip serology for electron microscopic identification of plant viruses. Virology 36: 152-155.

5. BRANDES, J. 1957. Eiire electronemicrovoscopsiche Schnell method zrim Nachiveis faden-undstabchenfor miger Viren, inbensonder in Kartoffeldun Kel Keimen. Nachrbl. deutsch Pflanzenschtz dienst (Braunschweig) 9:151-152.

6. BRLANSKY, R. H., and K. S. DERRICK. 1974. Serologically specific electron microscopy; detection and identification of tomato spotted wilt virus and icosahedral plant viruses in crude plant extracts. Proc. Am. Phytopathol. Soc. 1:21 (Abstr.).

7. COUCH, H. B. 1955. Studies on the seed transmission of lettuce mosaic virus. Phytopathology 45:63-69.

8. DERRICK, K. S. 1972. Immuno-specific grids for electron microscopy of plant viruses. Phytopathology 62:753 (Abstr.).

9. DERRICK, K. S. 1973. Detection and identification of plant viruses by serologically specific electron microscopy. Phytopathology 63:441 (Abstr.).

10. DERRICK, K. S. 1973. Quantitative assay for plant viruses using serologically specific electron microscopy. Virology 56:652-653.

11. DERRICK, K. S. 1975. Serological relationships among strains of sugarcane mosaic virus. Proc. Am. Phytopathol. Soc. 2:42 (Abstr.).

12. DERRICK, K. S., and R. H. BRLANSKY. 1974. Quantitative studies of potato virus Y using serologically specific electron microscopy. Proc. Am. Phytopathol. Soc. 1:20 (Abstr.).

13. DERRICK, K. S., and R. H. BRLANSKY. 1976. Assay for viruses and mycoplasmas using serologically specific electron microscopy. Phytopathology 66:815-820.

14. GOODING, G. V. JR. 1970. Natural serological strains of tobacco ringspot virus. Phytopathology 60:708-713.

15. MARROU, J., and C. M. MESSIAEN. 1967. The Chenopodium quinoa test: a critical method for detecting seed transmission of lettuce mosaic virus. Proc. Int. Seed Test. Assoc. 32:49-57.

16. McKINNEY, H. H. 1953. New evidence of viruses diseases in barley. Plant Dis. Rep. 37:292-295.

17. PENNY, J. B. JR., L. P. WEINER, R. M. HERNDON, O. NARAYAN, and R. T. JOHNSON. 1972. Virions from progressive multifocal leukencephalopathy; rapid serological identification by electron microscopy. Science 178:60-62.

18. STACE-SMITH, R. 1970. Tobacco ringspot virus. No. 17 in Descriptions of Plant Viruses. Commonw. Mycol. Inst., Assoc. Appl. Biol., Kerr, Surrey, England. 4 p.

19. STEERE, R. S. 1956. Purification and properties of tobacco ringspot virus. Phytopathology 46:60-69.

20. RYDER, E. J., and A. S. JOHNSON. 1974. A method for indexing lettuce seeds for seedborne lettuce mosaic virus by airstream separation of light from heavy seeds. Plant Dis. Rep. 58:1037-1039.

21. ZINK, F. W., R. G. GROGAN, and J. E. WELCH. 1956. The effect of the percentage of seed transmission upon subsequent spread of lettuce mosaic. Phytopathology 46:662-664.

VITA

Ronald Hillin Brlansky was born January 3, 1949, in Houston, Texas, the son of Evelyn Hillin Brlansky and John J. Brlansky. He was graduated from Robert E. Lee High School in May, 1966, and entered Texas A&M University in September, 1966. In May, 1970, he received a Bachelor of Science degree in zoology. He entered graduate school at Texas A&M University in plant pathology in September, 1971, and received a Master of Science degree in December, 1973.

Mr. Brlansky enrolled at Louisiana State University in August, 1973, and is presently a candidate for a Doctor of Philosophy degree in plant pathology.